Brit. J. vener. Dis. (1962), **38**, 78.

LOSS OF VIRULENCE *IN VITRO* OF MOTILE *TREPONEMA PALLIDUM**

BY

JOHN W. CLARK, JR.

From the Venereal Disease Experimental Laboratory, Communicable Disease Center, U.S. Public Health Service, School of Public Health, University of North Carolina, Chapel Hill, N.C.

Although *Treponema pallidum*, the causative agent of syphilis, has not been cultivated *in vitro*, the organism may remain motile under optimum conditions for periods up to 3 weeks. It has been demonstrated repeatedly that organisms maintained *in vitro* may cause syphilis when injected into rabbits. There are, however, conflicting statements in the literature on whether motile *T. pallida* are invariably virulent, *i.e.* whether all motile organisms can cause syphilis when injected into a suitable host. Since this question could have important implications in the problem of culturing the organisms, we are reporting experiments designed to give a definite answer.

Nelson (1948) stated that, when *T. pallidum* was maintained *in vitro* for periods of 54, 72, 144, or 192 hours at 30°C. and subsequently injected into rabbits, the animals developed darkfield-positive lesions. Further, when the number of viable organisms was calculated from the total count and the percentage of motile organisms, the incubation periods found were consistent with the ranges obtained by Magnuson, Eagle, and Fleischman (1948) with freshly isolated *T. pallidum*. This result suggested to Nelson that there was no loss of virulence when the organisms were maintained *in vitro*. It should be pointed out, however, that the standard deviation in the determination of the incubation period (as a function of number of organisms injected) is so large that only large differences between motility and virulence would be significant.

By contrast, Thompson, Greenberg, and Magnuson (1950), in studying the effect of the immobilizing antibody on the loss of infectiousness of *T. pallidum*, concluded that virulence might in fact be lost slightly before motility ceased. Weber (1960) (in a report published after the completion of the work described in the present paper) stated that there was a significant increase in the time required to produce darkfield-positive lesions in rabbits inoculated with motile *T. pallida* which had been maintained *in vitro* for 4 days or more.

There is also disagreement on whether organisms exposed to antisyphilitic drugs *in vitro* lose motility and virulence at the same rate. Eagle (1938, 1940) demonstrated that exposure to arsenic and bismuth compounds *in vitro* rendered *T. pallida* non-infectious, although a considerable number were still actively motile. Similar results were obtained by Dunham and Rake (1945) and by Viaseleva (1958) in their studies on the effect of penicillin on *T. pallidum in vitro*. Upon exposure to penicillin, culturable avirulent treponemes lost their ability to multiply before their ability to move (Eagle and Musselman, 1944). In contrast, Nell (1954) concluded that organisms immobilized by penicillin were no longer viable, but that motile *T. pallida* remained virulent.

Methods

In vitro suspensions of virulent *T. pallidum* were prepared by extracting sliced rabbit testicular syphilomata into a basal medium (developed by Magnuson and previously reported in a communication from this laboratory: Doak, Freedman, Clark, and Petit, 1958). This basal medium contains human serum ultrafiltrate, normal rabbit serum, phosphate buffer, sodium pyruvate, and physiological saline. The syphilomata were obtained from rabbits injected intratesticularly with virulent organisms 7 to 12 days previously. The extracts were centrifuged at 500 × G for 5 to 15 minutes to remove gross particulate matter. The supernatants, which contained huge numbers of actively motile organisms, were incubated in anaerobic jars in a nitrogen-carbon dioxide (95 : 5) atmosphere; the gas used was freed of oxygen by passage over heated copper. In certain cases an extract of fresh beef liver was added to the suspension; this markedly increased the survival time although the effect was not entirely reproducible. The beef liver extract was prepared by the

* Received for publication November 21, 1961.

method of Tauber and Petit (1952) for the isolation of crystalline catalase. The catalase was removed from the solution by centrifugation and the supernatant was added to the treponeme suspension. The percentage motility and the concentration of organisms were determined as previously described (Magnuson and others, 1948).

The infectivity of each suspension at various time intervals was tested by direct animal inoculation, either intracutaneously or intratesticularly, and the animals were followed clinically and serologically for 6 months. The popliteal lymph nodes of most of the rabbits which were negative at this time were removed, minced, and emulsified in a mixture of equal volumes of normal inactivated rabbit serum and physiological saline, and the suspension was injected intratesticularly into normal rabbits. If these animals remained negative for 3 months, the original suspension was considered to be non-infectious. All animals throughout this study were housed in air-conditioned quarters at approximately 22°C.

Results

In an early experiment, three rabbits failed to develop syphilis after intracutaneous inoculation with $1{\cdot}7 \times 10^5$ motile *T. pallida* from a suspension showing 92 per cent. motility after 6 days *in vitro* at 30°C. Magnuson and others (1948) have shown that, on intracutaneous injection, only two treponemes are required to infect one-half of the animals and about 500 treponemes to infect 90 per cent. Therefore, the $1{\cdot}7 \times 10^5$ motile *T. pallida* inocula in this experiment probably contained fewer than 500 virulent organisms.

Similar results were obtained under a variety of experimental conditions. Table I gives the full particulars of two typical experiments performed with treponemes maintained at 20°C. It is seen that there is a loss of virulence before complete loss of motility.

The results of a number of similar experiments are summarized in Table II. Each inoculum was adjusted to contain 2×10^5 *vigorously motile T. pallida*. This number of freshly-collected organisms produce darkfield-positive chancres routinely after $14{\cdot}3 \pm 4{\cdot}6$ days (Magnuson and others, 1948). In Table II the number of inoculation failures and the increase in incubation periods for the successful injections increase with the *in vitro* incubation of the *T. pallidum*.

TABLE I

EFFECT OF THE *IN VITRO* INCUBATION PERIOD ON THE INFECTIVITY OF *T. PALLIDUM*

Experiment No.	*In vitro* Incubation (days)	Total No. of *T. pallida* injected per site	Per cent. Motile	No. of *motile T. pallida* injected per site	Proportion of Sites Positive	Average No. of Days required for Development of Darkfield-positive Lesions
1	0	$2{\cdot}1 \times 10^5$	96	$2{\cdot}0 \times 10^5$	3/3	9·3
	2	$2{\cdot}8 \times 10^5$	72	$2{\cdot}0 \times 10^5$	3/3	11·7
	4	$3{\cdot}7 \times 10^5$	54	$2{\cdot}0 \times 10^5$	1/3	23
	6	$7{\cdot}7 \times 10^5$	26	$2{\cdot}0 \times 10^5$	1/3	23
	8	$21{\cdot}0 \times 10^5$	24	$5{\cdot}0 \times 10^5$	1/3	34
2	0	$2{\cdot}1 \times 10^5$	96	$2{\cdot}0 \times 10^5$	3/3	10
	2	$3{\cdot}0 \times 10^5$	66	$2{\cdot}0 \times 10^5$	3/3	15
	4	$5{\cdot}5 \times 10^5$	36	$2{\cdot}0 \times 10^5$	3/3	21·7
	6	$8{\cdot}4 \times 10^5$	24	$2{\cdot}0 \times 10^5$	0/3	—
	8	$15{\cdot}6 \times 10^5$	32	$5{\cdot}0 \times 10^5$	0/3	—

Suspensions of motile organisms were maintained *in vitro* at 20°C. At 0, 2, 4 and 6 days, 2×10^5 *motile* organisms, and at 8 days, 5×10^5 motile organisms per site, were injected intracutaneously in each of six rabbits. Five separate sites on the backs of these rabbits were used for the five inoculations and the two different suspensions, designated Experiments 1 and 2, were employed.

TABLE II

DECREASING INFECTIVITY OF *T. PALLIDUM* WITH INCREASING INCUBATION TIME *IN VITRO*

In vitro Incubation (days)	Total No. $\times 10^5$ *T. pallida* Injected per Site (Range)	Per cent. Motile (Range)	No. of *Motile T. pallida* Injected per Site	Proportion of Sites Positive	No. of Days Required for Development of Darkfield-positive Lesions (Range)
0	2·0	96–100	$2{\cdot}0 \times 10^5$	36/36	7–14
4	8·5 – 2·6	24–76	$2{\cdot}0 \times 10^5$	34/36	15–27
6	18·2 – 3·3	11–60	$2{\cdot}0 \times 10^5$	24/36	17–34
8	25 – 4·7	8–44	$2{\cdot}0 \times 10^5$	6/25	23–27
10	50 – 6·3	4–32	$2{\cdot}0 \times 10^5$	0/18	—
12	50 – 33	4–6	$2{\cdot}0 \times 10^5$	0/6	—

Suspensions of motile organisms were maintained *in vitro* at 20°C. *Motile organisms*, 2×10^5 per inoculation, were injected intracutaneously into the backs of rabbits, and the sites followed for the development of darkfield-positive lesions.

When *T. pallidum* is maintained at 35°C., rather than at 20°C. as reported in Tables I and II, the infectivity is also lost more rapidly than motility (Table III).

These results demonstrate the separation of virulence from motility. A more quantitative measure of virulence can be obtained by the use of graded inocula. Thus, Magnuson and others (1948) demonstrated that 2×10^5 freshly isolated motile organisms were invariably infective on intracutaneous inoculation, and that the percentage of positive lesions fell to 92, 93, 88, 71, and 47 per cent. as the number of motile organisms was reduced to 2×10^4, 2×10^3, 2×10^2, 2×10, and 2, respectively. Accordingly, if we obtain more than one failure in ten inoculations, we can say with confidence that less than 2×10^3 virulent organisms were injected. Table IV summarizes some experiments in which graded inocula were injected into rabbits after maintaining the organisms for 0, 2, and 4 days *in vitro*. After 4 days at 35°C., only four of nine inocula of 200,000 and two of nine inocula of 20,000 motile *T. pallida* produced lesions. The average *in vivo* incubation periods to darkfield positivity were 27·8 and 33 days respectively. It is apparent that less than 1 per cent. of the treponemes retaining characteristic motility after 4 days *in vitro* are infective.

It has been demonstrated (Magnuson and others, 1948) that with as few as ten freshly isolated motile organisms, 91 per cent. of intratesticular inoculations will produce darkfield-positive lesions, and that twenty organisms will give 100 per cent. successful results. Obviously, the intratesticular route is more sensitive than intracutaneous inoculation for judging the degree of infectiveness of *T. pallidum*. The results of two experiments where graded inocula of *T. pallidum* were injected intratesticularly are shown in Table V (opposite).

Such experiments differ from those described above in that a different rabbit must be used for each inoculation when intratesticular inoculation is employed. The results clearly demonstrate the separation of motility and virulence. Serological tests on the rabbits used in these experiments were consistent

TABLE III

LOSS OF INFECTIVITY OF *T. PALLIDUM* AFTER *IN VITRO* INCUBATION at 35°C

In Vitro Incubation (days)	Total No. of *T. pallida* injected per site	Per cent. Motile	No. of *motile T. pallida* injected per site	Proportion of Sites Positive	Average No. of Days required for Development of Darkfield-positive Lesions
0	$1{\cdot}00 \times 10^4$	100	$1{\cdot}00 \times 10^4$	3/3	7
	$2{\cdot}00 \times 10^7$		$2{\cdot}00 \times 10^7$	3/3	4
2	$2{\cdot}40 \times 10^4$	54	$1{\cdot}30 \times 10^4$	3/3	13
	$2{\cdot}40 \times 10^6$		$1{\cdot}30 \times 10^6$	3/3	7
3	$1{\cdot}96 \times 10^4$	51	$1{\cdot}00 \times 10^4$	0/3	—
	$1{\cdot}96 \times 10^6$		$1{\cdot}00 \times 10^6$	0/3	—

A suspension of motile organisms was maintained *in vitro* at 35°C.; motility was lost completely after 5 days. Three rabbits were inoculated intracutaneously at 0, 2, and 3 days.

TABLE IV

EFFECT OF NUMBER OF *MOTILE T. PALLIDA* INOCULATED INTRACUTANEOUSLY AND *IN VITRO* INCUBATION PERIOD ON INFECTIVITY OF *T. PALLIDUM*

Experiment No.	*In vitro* Incubation (days)	Per cent. Motile	Average No. of Days Required for Development of Darkfield-positive Lesions after Injection of Various Numbers of *Motile T. pallida*					
			2×10^5	2×10^4	2×10^3	2×10^2	2×10	2
1	0	100	7·8	12·6	14·6	16·4	20·8	22·8
	4	78	27·8 (4/5)	33 (2/5)	(0/5)	(0/5)	(0/5)	(0/5)
	7	21	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)	(0/5)
2	0	100	7·8	9·2	10·6	16·0	20·3 (4/5)	(0/5)
	2	58	20·3	22·2	22 (4/5)	27·4	32 (2/5)	(0/5)
3	0	100	8·2	11·2	15·8	17·2	21·5 (4/5)	22·6
	2	72	14·2	20·8	21·8	27·8	26 (1/5)	26 (1/5)
	4	48	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)

Three suspensions of motile treponemes (designated Experiments 1, 2, and 3) were maintained *in vitro* at 35°C. At intervals, graded inocula from these suspensions were injected intracutaneously into rabbits. Six sites were used on the back of each rabbit, one site for each inoculum. Each inoculum indicated above was injected on each day noted into five rabbits except on Day 4, Experiment 3, when only four rabbits were used. The numbers in parentheses give the proportion of positive sites in instances in which some inoculations did not infect.

TABLE V

EFFECT OF NUMBER OF *MOTILE T. PALLIDA* INOCULATED INTRATESTICULARLY AND *IN VITRO* INCUBATION PERIOD ON INFECTIVITY OF *T. PALLIDUM*

Experiment No.	*In vitro* Incubation (days)	Per cent. Motile	Days Required for Development of Darkfield-positive Lesions after Injection of Various numbers of *Motile T. pallida*					
			10^6	10^5	10^4	10^3	10^2	10
1	0	97	11	15	22	20	27	29
	1	93	17	17	19	24	60	45
	2	81	18	20	25	27	neg.	neg.
	3	31	22	26	29	neg.	neg.	neg.
2	0	100	13	15	17	20	24	29
	1	48	12	14	23	28	33	35
	2	50	15	20	27	32	32	34
	3	25	17	24	32	31	neg.	neg.

Suspensions of motile organisms were maintained *in vitro* at 35°C. At 0, 1, 2, and 3 days, graded inocula were injected intratesticularly into each of 48 rabbits. One rabbit was used for each inoculation and one testis only of each rabbit was inoculated. Two separate suspensions, designated Experiments 1 and 2, were employed.

with the clinical results. All 48 rabbits were VDRL negative initially. 60 days after inoculation all dark-field-positive animals were VDRL-positive, except for one animal in each experiment, both of which were weakly reactive. The sera of the seven rabbits with no clinical symptoms were VDRL-negative.

Discussion

The experiments reported in this paper clearly demonstrate that virulence may be lost earlier than motility, *i.e.* motile treponemes are not necessarily able to produce disease. It should be emphasized that neither the percentage motility nor the *in vitro* incubation period can be used as an index of the infectivity of a suspension of treponemes. In one experiment (cf. Table V) a suspension which had been maintained *in vitro* for 3 days and which exhibited 25 per cent. motility was able to cause syphilis if 10^3 or more motile treponemes were inoculated intratesticularly. In a similar experiment (not given in the Tables), we found that, after the same incubation period, 7×10^6 motile organisms from a suspension 51 per cent. motile were not infectious. There was, therefore, at least a 7,000-fold difference in the virulence of these two suspensions.

Maintenance of virulence may be a useful criterion in *in vitro* culture of *T. pallidum* and in determining the *in vitro* effects of anti-treponemal agents. Further work has been planned in an attempt to elucidate the factor or factors responsible for the loss of virulence of *T. pallidum* maintained *in vitro*.

Summary

Under the conditions studied, *T. pallidum*, maintained *in vitro*, lost virulence at a greater rate than motility. Loss of ability to infect was demonstrated by two facts:

(1) The increase in the *in vivo* incubation periods of quantitated inocula of motile organisms with the increase of time maintained *in vitro*.

(2) More particularly, the failure of older suspensions of *motile T. pallida* to infect.

REFERENCES

Doak, G. O., Freedman, L. D., Clark, J. W., Jr., and Petit, E. L. (1958). *Antibiot. and Chemother.*, **8,** 342.

Dunham, W. B., and Rake, G. (1945). *Amer. J. Syph.*, **29,** 214.

Eagle, H. (1938). *J. Pharm. exp. Ther.*, **64,** 164.

—— (1940). *Ibid.*, **69,** 342.

—— and Musselman, A. D. (1944). *J. exp. med.*, **80,** 493.

Magnuson, H. J., Eagle, H., and Fleischman, R. (1948). *Amer. J. Syph.*, **32,** 1.

Nell, E. E. (1954). *Ibid.*, **38,** 92.

Nelson, R. A. (1948). *Amer. J. Hyg.*, **48,** 120.

Tauber, H., and Petit, E. L. (1952). *J. biol. Chem.*, **195,** 703.

Thompson, F. A., Jr., Greenberg, B. G., and Magnuson, H. J. (1950). *J. Bact.*, **60,** 473.

Viaseleva, S. M. (1958). *Z. Mikrobiol.* (*Mosk.*), **29,** No. 7, p. 47.

Weber, M. M. (1960). *Amer. J. Hyg.*, **71,** 401.

Perte de puissance *in vitro* du *Treponema pallidum* mobile

RÉSUMÉ

Dans les circonstances de cette étude, le *T. pallidum*, soutenu *in vitro*, a perdu sa puissance plus vite que sa mobilité. L'absence d'infectiosité était démontrée par deux faits:

(1) La période d'incubation *in vivo* d'inocula quantitatifs des organismes mobiles augmentait avec la période du soutien *in vitro*.

(2) Les suspensions plus âgées de *T. pallidum* mobile manquaient à transmettre l'infection.